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In Vitro Models and Methods for Bioassay and Studies of Cellular Mechanisms*

Relationship Between *In Vivo* and *In Vitro* Testing

This report discusses the use of *in vitro* systems to detect metal carcinogens and to provide information on the mechanisms of metal carcinogenesis. The use of *in vivo* systems is also discussed, where they may provide additional information. Even though no single system has proven adequate for the definitive identification of all metal mutagens and carcinogens, the aggregate results of these studies indicate that the combined use of several systems may provide a valuable, initial screen for metals and their compounds. It is now imperative to evaluate more complex aggregates and mixtures of metals and their compounds that are environmentally ubiquitous (*1*). Metals appear to behave similarly to other carcinogens in cell transformation tests; however, they are frequently negative in bacterial mutagenesis revertant assays.

In addition to rapid screening, cellular systems are particularly advantageous for analyzing the mechanism of metal mutagenesis and carcinogenesis. It is only in such systems that one may be able to study the mechanism and specificity of metal-induced cellular alterations which may be causative factors in neoplasia. The physiological chemistry of different metals may vary widely. For example, arsenic and chromium may exist in solution as anions, whereas most other carcinogenic metals exist principally as cations.

A major difficulty with metal carcinogens is that no specific metal-nucleic acid interactions analogous to those obtained with organic chemical carcinogens and particular loci in DNA molecules have been observed *in vivo*. Although recent evidence suggests that most metals do not induce repair synthesis, studies using bacterial or eucaryotic mutants deficient in DNA repair pathways should provide valuable information on metal induced alterations and mechanisms of repair. Furthermore, these cellular assays provide an experimental setting for tracing the involvement of mutations in carcinogenesis. In addition, cell-free systems have permitted measurements of the effects of metals on the fidelity of DNA synthesis with the ultimate goal of analyzing in molecular terms the mechanism of metal carcinogenesis. Conversely, an understanding of metal-cellular interactions should provide new systems to more adequately evaluate the toxic, mutagenic and carcinogenic effects of metals common in our environment. In view of these difficulties and future prospects, recommendations are made in this report and in the commissioned papers to provide more information which will lead to an understanding of the mechanisms of metal carcinogenesis.

Bacterial Mutagenesis

Summary of Present Knowledge

To date, bacterial mutagenesis systems have not been successful in predicting the carcinogenicity of

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metals. The strongest mutagenic effects in bacterial systems are found with certain square planar platinum compounds, e.g., *cis*-dichlorodiammine platinum (II) (*2*, *3*). There is also evidence for the mutagenicity of chromium, selenium, and manganese compounds (*4*). Reports of positive results with other metal compounds are incomplete or have not been reproduced. Thus, as they are commonly used, bacterial mutagenesis systems are not adequate for screening metals but may be useful for studying the mutagenic mechanisms of those metals which give positive responses.

Chromate mutagenesis can be most easily detected by the fluctuation test (*5*, *6*), in which multiple rounds of cell divisions occur in the presence of nontoxic metal concentrations. A reasonable hypothesis is that chromate mutagenesis occurs via a decrease in the fidelity of DNA replication. If other metal compounds act through similar mechanisms, mutagenic effects might also be detected by the fluctuation test. However, arsenite and nickel were negative in this assay (*5-7*).

Bacterial mutagenesis assays may be useful in screening for comutagenic effects of metal compounds. Since many metal ions have been shown to act as enzyme inhibitors, enzymes involved in DNA repair may be particularly susceptible to certain metal compounds at subtoxic doses. These compounds may act as comutagens by inhibiting repair of DNA. Arsenic has been shown to inhibit DNA repair in *E. coli* (*8*) and to enhance mutagenesis by ultraviolet light (T. Rossman, unpublished observation). In performing such studies, a variety of mutagenic agents requiring different modes of DNA repair should be employed. The availability of bacterial mutants defective in a variety of DNA repair pathways make this system useful for pinpointing mechanisms of mutagenesis or comutagenesis (*9*). The use of selective toxicity on repair-deficient mutants as a screen for DNA damaging agents may prove valid for metals if it can be demonstrated that (a) exposure to metal compounds does in fact result in repairable damage to DNA; and (b) the mutation in the repair-deficient strain has no pleiotrophic effects which would result in reduced viability under general stress. Another bacterial assay system which might be useful in studying the mechanism of action of metal compounds is the induction of lambda prophage (*10*). Most agents which damage DNA cause the induction of prophage to the active viral form, resulting in viral replication and lysis of the host cell. Mutagens which interact with DNA polymerase to cause decreased fidelity would not be expected to induce prophage, whereas agents which cause base damage of DNA strand breaks would act as prophage inducers. This system might be particularly useful in cases where free radicals and peroxides are generated.

Mammalian Cell Mutagenesis

Much more needs to be known about metal mutagenesis in mammalian cells. The use of mammalian cells in metal mutagenesis studies will yield important information with regard to insoluble particulates which might not penetrate bacteria but could possibly enter mammalian cells by pinocytosis, and then be converted to an active species. In addition, as in the case with bacterial systems, comutagenesis assays could also be carried out in mammalian cells.

Chinese hamster lines (*11*) (CHO and V79) and the L5178y mouse lymphoma line are useful for such studies (*12*). Available genetic loci include Na-K ATPase, HGPRT, and thymidine kinase. In addition, in order to facilitate mechanistic studies, reversion assays should be developed to distinguish between base-pair substitutions and frameshift mutations. As in the case for bacterial mutagenesis, various metallic species can be used in both long term exposure to nontoxic concentrations (as in a fluctuation test) as well as short term exposure to higher doses. Significant increases in the absolute number of mutants over the control is preferable to derived values (mutants/survivors) in determining whether a compound is a mutagen.

Because the primary targets for metals and other carcinogens are often epithelial cells, and because epithelial cells may differ from fibroblasts as to the uptake and metabolism of metal compounds, mutagenesis systems using epithelial cells from various organs are needed.

In Vitro Cell Transformation

Quantitative *in vitro* transformation assays for the evaluation of the potential carcinogenic activity of metals are available using mammalian cell cultures. These assays involve (a) transformation of hamster or mouse cells following exposure to metal salts (*13*) or (b) enhancement of viral transformation of cells following treatment with chemicals (*14*).

Direct Chemical Transformation

Induction of morphologic transformation by organic chemical carcinogens has been demonstrated in a variety of mammalian cells (*15*) and by the inorganic metal salts in Syrian hamster embryo cells. A number of other cell systems are currently

being used to screen for potential carcinogens and to study the processes of initiation, expression and promotion. The mouse cell lines Balb 3T3 and C3H10T½ have not been extensively used with metal carcinogens. The Syrian hamster embryo cell assay is suitable to study cell transformation induced by metal salts. These cells are diploid, retain metabolic function when used either freshly prepared or as frozen stocks, undergo progressive events leading to expression of the neoplastic state, and have very low frequency of spontaneous transformation. Furthermore, tumorigenicity is observed following the injection of these transformed cells into random-bred newborn or weanling Syrian hamsters. Assays are conducted by scoring transformed colonies among surviving colonies or as foci against a confluent background of normal cells.

Metaplasia, hyperplasia and other phenomena which are tissue specific may play an important role in metal carcinogenesis. In addition, transformation assays should be attempted in epithelial systems (*16*), such as those now being developed for skin (*17*) and liver cells (*18*). The possible use of organ fragments, in which histologic organization is preserved, could also be explored.

Viral-Chemical Interactions

A sensitive assay for the demonstration of carcinogenic potential of metal compounds is the enhancement of SA7 virus-induced transformation of hamster embryo cells. This test implies that agents capable of causing direct or indirect damage to cellular DNA will increase the frequency of morphological transformation by increasing the integration of viral DNA into cellular DNA at the damage site. The assay has responded to a wide variety of chemicals including known carcinogenic metal compounds (*14*). The assay may be used to obtain useful information regarding cooperative action between metals (synergistic or cocarcinogenic activity); inhibition of activity by other metals; and, effective exposure time required for optimal biologic activity. The temporal relationship between metal and virus addition provides an indication of the persistence of DNA damage induced by metal treatment, e.g., rapid disappearance of enhancing activity may be indicative of repair of damage, whereas persistence of enhancement indicates that the damage remains. The sequence of addition of virus and chemical in enhancement assays may also provide some information concerning the mechanism of action of metal carcinogens. Carcinogens found to interact directly with cellular DNA will increase the viral transformation frequency when added before or immediately after DNA-virus inoculation; some carcinogens that do not appear to directly damage or bind to DNA are found to be more active when added 5-7 hr after virus addition and cell transfer. The latter observation suggests that enhancement may occur as a result of interference with scheduled cell DNA synthesis during the time that viral DNA is being processed into cellular DNA, the interference may allow a greater opportunity for viral DNA to be integrated.

Cell-Free Systems

Cell-free bioassays may be developed as screening systems for indication of metal mutagens/carcinogens (*19*, *20*). This has been demonstrated by the observations of Sirover and Loeb (*20*, *21*) and others (*22*) that mutagenic/carcinogenic metals decrease the fidelity of *in vitro* DNA synthesis or synthetic polynucleotide templates. These studies have been further supported in experiments using DNA polymerases from a variety of sources, and most recently by the development of a bioassay system which measures the fidelity by which a natural template (from bacteriophage ϕX174) is copied *in vitro* (*23*).

Perhaps a more important aspect of cell-free bioassays is the possibility of examining some of the putative mechanisms of metal mutagenesis/carcinogenesis. In cell-free systems, homogeneous reaction components and conditions are defined and thus can be systematically varied. For example, DNA replication can be examined in the absence of DNA repair, and the contribution of each of the proteins involved in these processes can be determined. Furthermore, using such systems, one may begin to analyze the effects of metals at the molecular level. Metal ions may alter the fidelity of DNA synthesis by interacting with nucleotide substrates, the DNA polymerase, or with the DNA template. An analysis of the changes in the nucleotide sequence of DNA products copied from natural DNA templates in the presence of various metal ions and/or biased nucleotide pools should provide more definitive results concerning specificity of metal-nucleotide and metal-template interactions. Studies with *E. coli* and bacteriophage T_4 indicate that DNA replication proceeds by the concerted action of multiple proteins. Using the ϕX174 assay system, it has been possible to measure the contribution of various replication proteins to fidelity (*24*). Thus, the effects of metals on the accuracy of DNA replication can be assayed in a cell-free system that begins to approach the complexity involved in chromosomal DNA replication in cells.

The aforementioned studies are based on the

hypothesis that the mutagenic/carcinogenic effects of metals result in the permanent alteration of the DNA during DNA replication (somatic mutation hypothesis). Alteration in reactants could be in template, substrate, or enzyme. An alternative mechanism of metal mutagenesis/carcinogenesis may involve the alteration of gene expression, which can also be examined in cell-free systems. One study (*25*) indicates that chain initiation of RNA synthesis was stimulated by certain mutagenic metals and was inhibited by several nonmutagenic metals. Limited studies (*25*) have suggested that metal ions may also cause errors in RNA synthesis. These phenomena, like metal induced infidelity of DNA synthesis, could be a result of metal interactions with the DNA template. Regulation of gene expression could also occur at the level of the cellular proteins, and aspects of this may be examined in cell-free systems. For example, a study (*26*) on the fidelity of cell-free protein synthesis demonstrated that high Mg concentrations increased the incorporation of incorrect amino acids into protein.

Another method of interest is the affect of metals on mixing curves of synthetic polyribonucleotides (*27*). Manganese and cadmium induced mispairing, whereas magnesium and zinc did not.

Chromosome Aberration, Sister-Chromatide Exchange and Micronucleus Tests

The metaphase chromosome aberrations produced by metals represent one of the few practical methods currently available for evaluating prior genetic damage in man. The induction of chromosome aberrations by metal compounds has been extensively studied in cultured mammalian cells (*4*, *19*, *28*). There is a weak correlation between the frequency of chromosome aberrations and the carcinogenicity of metal compounds. The major problem appears to be the number of false positive results obtained in studies in cultured cells. This may occur because the assay has not been optimized for the detection of carcinogenic metals or because the observed chromosome aberrations are not directly related to carcinogenesis.

Two additional assays, sister-chromatid exchange (SCE) (*28-32*) and micronucleus (extranuclear interphase chromosome fragments) (*33-35*), are increasingly being used in cytogenetic studies in man and in experimental systems. Their potential advantages over chromosome aberrations are easier enumeration and frequent detection at lower doses of carcinogen. However, there are few published data on the production of SCEs or micronuclei by metal compounds (*36*, *37*). Their significance as indicators of genetic damage is also not established.

All three assays require validation and optimization for the specific detection of metal carcinogens/mutagens. For studies *in vitro*, the major parameters to be examined are: treatment time (acute and chronic), sample time, cell type (lymphocytes, cell lines, primary cultures), specificity (whether cytogenic damage is seen at nontoxic doses or only near the toxic limit), treatment medium (buffers, culture fluid, serum), and role of chelating agents. All three assays could be performed in the same cell population. This will allow a direct comparison of the strengths and weaknesses of each assay and may indicate suitable combinations to be used in routine analysis.

The assays also require validation *in vivo*. Again, treatment time, sample time, and a variety of cell types, e.g., lymphocytes, bone marrow cells, germ cells, should be sampled and examined. Comparison of experimental results obtained *in vitro* may help to determine the significance of positive and negative results in human studies.

Studies on the Mechanism of Chromium Carcinogenesis

We have selected chromium as an example of a metal in which detailed investigations of mechanisms may yield results relevant to carcinogenesis. Animal cancer studies implicate Cr(VI) as the biologically active species in carcinogenesis. This parallels the behavior in *in vitro* tests (e.g., mutagenicity in the Ames Salmonella assay), while Cr(III) compounds prove more active in cell-free bioassays of genetic toxicity (*38*, *39*). These results are explicable in terms of the uptake-reduction model of Jennette (*40*). According to the model: chromium enters the cell as the hexavalent species, perhaps using the sulfate transport system (*41*). Once inside the cell, microsomal enzymes, including those of the P450 system, reduce hexavalent chromium to trivalent chromium, which then reacts with critical intracellular sites to induce genotoxic effects. Recent data further suggest that reduced chromium may bind directly to the nucleotide bases in DNA (*41*). Thus, in the case of chromium, evidence from *in vitro* tests consistently suggest that carcinogenesis may involve mutagenic initiation of somatic cells via direct DNA interactions. The hypothesis that chromium is a mutagenic initiator may now be explored by a variety of procedures to further elucidate the biochemical mechanisms involved in its carcinogenicity. This could lead to an understanding of how one metal

acts as a carcinogen and would facilitate comparable studies on other metals.

Role of Free Radicals in Metal Carcinogenesis

There is considerable evidence that free radicals generated by ionizing radiation or organic chemicals play a role in carcinogenesis (*42*, *43*). Many transition metals, acting either as metal-enzymes or in a complexed form, catalyze the oxidation of endogenous or exogenous chemicals to produce peroxides, epoxides, and free radicals such as superoxide and hydroxyl (*44*). Many of these species damage and inactivate DNA (*45*, *46*), are mutagenic (*47-49*), and may be carcinogenic (*44*, *50*). The involvement of free radicals in metal carcinogenesis/mutagenesis, therefore, deserves further investigation. These studies should be conducted at the molecular and cellular levels.

Integration of *In Vitro* Bioassays into Epidemiological Cancer Studies

Present State of Knowledge

In order to validate the use of *in vitro* tests as predictive indicators of carcinogenesis, the test results must be integrated into epidemiological cancer studies. Interdisciplinary investigations of this nature are now in progress (*51*). Such studies attempt to identify the significant factors contributing to excess cancer rates in both occupational and community-wide settings. Environmental and biological samples are analyzed using *in vitro* assays and chemical methods. These measurements will provide baseline information for both prospective and retrospective epidemiological studies designed to evaluate the contribution of metals and other environmental factors in the etiology of cancer.

General Conclusion and Recommendations

The *in vitro* assays provide excellent model systems for the study of metal carcinogenesis. The various areas amenable to research include: (a) molecular analysis of mechanisms of metal mutagenesis at the level of DNA replication and repair; (b) determination of those agents that may require promotion for carcinogenic activity; (c) synergistic action between metals, including syncarcinogenesis and cocarcinogenesis; (d) antagonistic activity between metals; (e) prerequisite exposure periods for demonstration of biologic activity; (f) assay of gene products in the same cells treated with various metal salts.

Recommendations for Future Research

Bacterial Mutagenesis

The fluctuation test should be used in testing metals and their compounds for mutagenesis. Toxic doses should be used for short time periods and dose- dependent increases in the absolute number of mutants per plate should be observed before a compound is reported to be a mutagen.

Strains of bacteria defective in mismatch repair (*52*, *53*) should be evaluated as possible indicator strains for metal mutagens, since this repair system may eliminate errors in DNA replicated in the presence of metal.

The ability of metals and their compounds to induce lambda prophage should be tested. This would be useful in studies on the mechanism of metal mutagenicity and carcinogenicity.

Mammalian Cell Mutagenesis

A number of mammalian cell lines and genetic markers should be used to assay for mutagenicity by metal compounds. Chinese hamster (CHO, V79) and mouse lymphoma (L51758y) cell lines, with available genetic loci (Na, K-ATPase, HGPRT, and thymidine kinase), are recommended.

Mutagenesis systems using epithelial cells from various organs should be developed.

Cell Transformation

In vitro transformation by metal compounds should be studied with the established Syrian hamster and mouse (Balb 3T3, C3H10T½) cell lines, and with epithelial cells (e.g., skin, liver) being developed for such studies.

The enhancement of virus-induced transformation of hamster embryo cells by metal compounds should be further explored to provide information on metal-virus-gene interactions.

Cell-Free Systems

Further exploration of metal decreased fidelity of DNA synthesis would be valuable in screening for

metal carcinogens and in determining mechanisms of metal carcinogenesis/mutagenesis.

Studies with a natural template (from bacteriophage ϕX174), in addition to synthetic polynucleotides, would be especially useful in analyzing the effects at the molecular level.

Additional studies on RNA and protein synthesis would provide further information on the effects of metals on gene expression.

The effects of metals on mixing curves of synthetic polyribonucleotides should be studied.

Chromosome Aberrations

Increased frequencies of chromosome abnormalities are suggestive, but not proof, of an increased risk of cancer. Studies of chromosome abnormalities in persons exposed to known or suspected carcinogens, properly controlled for age and characterized for identity, level and duration of exposure, can prove useful for both research and public health purposes (*19*, *28*).

The finding of increased levels of chromosome aberrations or SCEs in a group of exposed persons (in the absence of confounding factors such as smoking) is cause to suggest: reduction in exposure to suspected agents, characterization of suspected agents, and surveillance of the exposed group for chromosome abnormalities, morbidity and mortality.

Chromosomal aberrations, SCE, and micronucleus formations should be measured in animals involved in carcinogenicity bioassays.

Assay for nondisjunction should be developed in man. Nondisjunction generates aneuploidy in man but cannot be determined by standard cytogenetic techniques. New techniques, such as variation in DNA content of sperm are promising, but require validation.

Criteria for acceptable results should be determined in assays. Minimal standards for acceptability are: enumeration of types of aberrations, multiple sample times, scoring of at least 100 metaphases for chromosome aberrations, or 30 metaphases for SCE.

Mechanism of Chromium Carcinogenesis

Chromium metabolism in bacteria and animal cells in culture should be studied.

Effects of organochromium complexes, both *in vitro* and in whole animal bioassays, should be analyzed.

The mechanism of chromium mutagenesis and oncogenic transformation of animal cells in culture should be investigated.

The initiating and promoting activity in skin tumor sensitive (Sencar) mice (*54*) and in cells *in vitro* (*55*) should be determined.

The distribution and binding of chromium to cellular organelles and macromolecules should be studied.

Chromium effects on the cell-free synthesis of DNA, RNA and protein, with particular emphasis on fidelity, should be analyzed.

Cr(III)-DNA adducts and damage to DNA caused by Cr should be characterized.

Role of Free Radicals

The presence of free radicals in metal treated cells and cell-free systems should be determined by electron spin resonance spectroscopy using spin traps (*56*).

The effects of free radicals should be determined by inhibition of metal mediated events using catalase, superoxide dismutase, and free radical scavengers.

Integration of *In Vitro* Bioassays into Epidemiological Cancer Studies

Integration of *in vitro* bioassays into epidemiological studies of metal carcinogenesis can provide useful comparisons. Such studies could be carried out among workers occupationally exposed to metal carcinogens. Welders, for example, are exposed to carcinogenic chromium compounds in the stainless steel fumes generated during arc welding operations. Epidemiological and cytogenetic studies of groups of exposed persons could be integrated with studies of samples collected from the workplace. In addition to physical and chemical analysis, workplace samples could also be analyzed for mutagenic activity using *in vitro* tests.

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